NMR STUDIES OF H_2O:*N*-PHENYL (*N*-PHENYL-β-D-GLUCOPYRANOSYLAMINE)URONAMIDE INTERACTIONS IN DIMETHYL SULFOXIDE AT TWO FIELDS

ABSTRACT

During the course of NMR structural investigations of the title compound (*N*-phenyl uronamide) we noticed the presence of a strong H_2O activity-dependent NOE between the small amount of H_2O associated with the carbohydrate's hydroxyl protons in solutions of DMSO-d_6. The -OH/H_2O off-diagonal t_1 slices displayed a NOESY τ_m-dependence similar to molecules (τ_c = 0.1-5 ns) experiencing slow exchange (1-10 s^{-1}). From T_1 data at two fields a τ_c for the title compound was calculated to be ca. 0.54 ns at 313 K. The -OH/H_2O exchange rate constant, κ, increased from 0.32 to 11.14 s^{-1} as the molar ratio of [H_2O]:[*N*-phenyl uronamide] increased from ca. 4.5 to 5.2. The latter finding indicated that the -OH/H_2O proton exchange process, which is strongly affected by the translational diffusion of H_2O, diminished as [H_2O] approached that which was inherently a complex in the crystalline structure (e.g., $C_{18}H_{20}O_5N_2 \cdot 4H_2O$) and was, presumably, tightly hydrogen-bound to the -OH/NH funtional groups. To test this, the title compound was recrystallized from ethanol/2,2-dimethoxypropane whereupon the bound H_2O was eliminated; the lack of H_2O in-

duced a significant upfield shift in the resonance frequencies of all the exchangable (-OH $\Delta\delta$=86.33Hz; -NH $\Delta\delta$=67.84Hz) functional groups relative to the methine protons (CH $\Delta\delta$=0.25Hz).

INTRODUCTION

Knowledge about the interactions between simple sugars and hydrogen bound H_2O is important because physicochemically unique characteristics of carbohydrates are imparted by the interactions between these biologically active compounds and their solvating species. DMSO makes an ideal milieu for understanding these interactions because carbohydrates retain much of their H_2O-induced conformation[1] in DMSO and one can specifically observe, assign and study the carbohydrate's -OH groups, and associated interactions, with small quantities of H_2O because their resonance frequencies are so disparate.

In this manuscript we present rotating-frame Overhauser enhancement spectroscopy (ROESY), NOESY and chemical shift evidence that *N*-phenyl (*N*-phenyl-β-**D**-glucopyranosylamine)-uronamide's (*N*-phenyl uronamide; Figure 1) H_2O's of crystallization are tightly bound to the -OH and -NH functional groups of the sugar moiety even after extreme dilution with DMSO.

RESULTS AND DISCUSSION

Previously,[2] we reported on the synthesis and structural characterization of *N*-phenyl uronamide. This acid sugar derivative is an unusual by-product of the activation of glucuronic acid's carboxyl group with a carbodiimide reagent[3-6] in the presence of the aromatic nucleophile, aniline. Originally,[2] we misassigned the H_2O resonance as the CH_3 of DMSO because we had assumed that the title compound was unstable in solution in the presence of free H_2O. Other studies were performed because we supposed the observed cross peaks between the -OH groups of the title compound and the "solvent" were due to magnetization transfer via some engaging phenomenon such as spin diffusion or 2nd order Overhauser

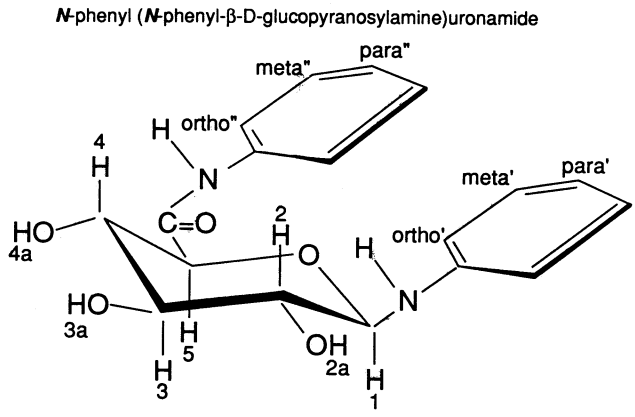

FIG. 1. Structure and conformation ($^4C_1$) of the title compound with ^1H position labels.

effects. However, upon recrystallizing the title compound in the presence of 2,2-dimethoxypropane (e.g., H_2O + 2,2-dimethoxypropane $\xrightarrow{\Delta}$ 2MeOH + acetone; Figure 2) we discovered that the "solvent" peak disappeared and, therefore, was in fact a small amount of H_2O which co-crystallized with the solute and/or which was adsorbed from the head-space above the solvent (ca. 30 mM). When *N*-phenyl uronamide was recrystallized, as described previously,[2] from hot EtOH without 2,2-dimethoxypropane and examined via ^1H NMR, using "100%" DMSO-d_6 which was taken from a freshly-opened ampule just prior to performing the experiment, the H_2Os of crystallization were found to exist in a ca. 4:1 molar ratio to the title compound; upon vacuum drying at ca. 100 °C the level of hydration was reduced to 1 H_2O:*N*-phenyl uronamide as measured by elemental analysis (Anal. Calcd for $C_{18}H_{20}O_5N_2 \cdot H_2O$: C, 59.66; H, 6.12; N, 7.73. Found: C, 59.67; H, 6.53; N, 7.39).[2] Unfortunately, similar misinterpretations may have been made on other small carbohydrates, such as cellobiose, inasmuch as comparable "DMSO"/-OH interactions, via NOESY NMR, have been promulgated.[1] We believe that H_2O/-OH exchange may have mistakenly been explained as a DMSO/-OH 1st or 2nd order

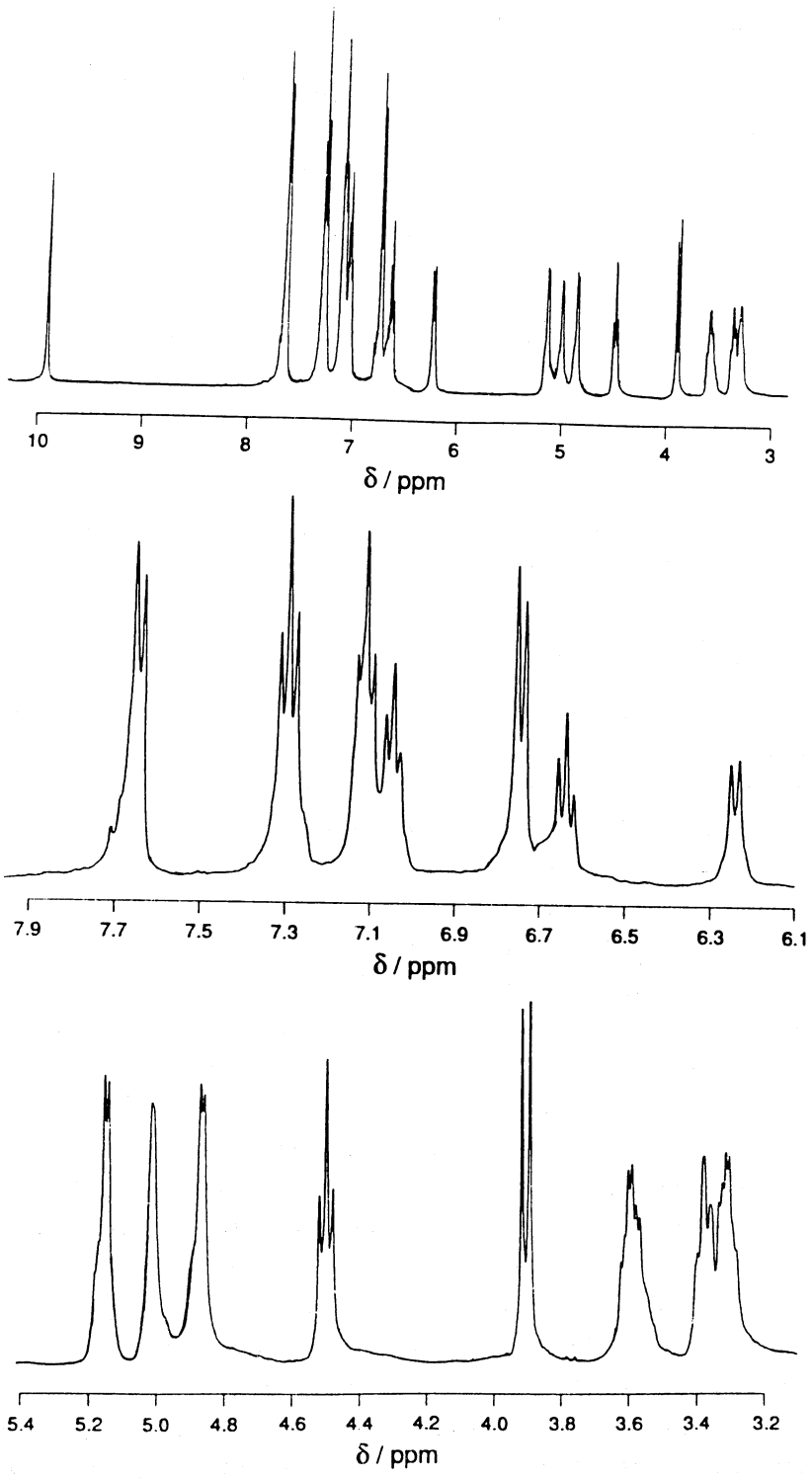

FIG. 2. ^{1}H NMR spectra of the title compound in the anhydrous state.

Overhauser effect via 2D NOE NMR spectroscopy in the aforecited[1] case.

In 2D Overhauser enhancement spectroscopic experiments, cross peaks not associated with scalar or J-coupling result from either direct cross relaxation[7,8] or exchange phenomena.[7-10] Direct cross relaxation (e.g., a "1st order Overhauser effect")[11] is a simple through-space dipolar spin-spin interaction proportional to the inverse 6th power of the distance between the interacting spin systems. Exchange-like phenomena,[7-15] while not as conceptually unambiguous as direct cross relaxation, can nevertheless be outlined as follows:

1. chemical equilibrium processes:
   a. chemical exchange[7-10]
   b. stereochemical exchange[9]
   c. relayed exchange[10] (results from apparent chemical exchange between non-exchangable ^{1}Hs and a solvating compound, such as H_2O; due to a magnetization exchange process between ^{1}Hs at an exchangable near-neighbor site and non-exchangable ^{1}Hs)
2. magnetization equilibrium processes:
   a. "2nd order Overhauser effects" [$\nu_o\tau_c \sim 1\text{-}10$][11,13-15]
   b. "spatial" and "spectral" spin diffusion [$\nu_o\tau_c \gg 10$][12]

The 2nd order Overhauser effect and other spin diffusion-like processes frequently occur in solids or solid-like solutions of macromolecules at high magnetic fields where simple spin diffusion becomes efficient. Cellobiose, whose molecular weight (mw = 342.29) is similar to the title compound (mw = 344.36), is certainly an unlikely candidate for any of the magnetization equilibrium processes enumerated above. In order to observe direct cross relaxation, the residence time of molecular interaction between the "solvent" and the cellobiose -OH groups would have to have been inordinately long.

For true chemical exchange processes information can be obtained about the dynamics of exchange from the mathematical behavior of the off-diagonal NOESY resonances as τ_m is increased. For instance, during very slow chemical exchange between some ^1H donor (e.g., an amide-H, τ_c = 0.1-5 ns)[10] and the solvating species, H_2O, cross peak integrals (I) should increase with mixing time (τ_m) in NOESY or ROESY experiments as a typical 1st order rate process where

$$I = I_o\{1 - e^{-K\tau_m}\} \quad (1)$$

and

$$I_o = \lim_{\tau_m \to \infty} I \quad (2);$$

in these relations I increases to a maximum, I_o, as a function of τ_m and eventually levels off; K is the 1st order rate constant in units of $\{\tau_m\}^{-1}$. For ^1H donor species, similarly-sized (τ_c = 0.1-5 ns) to the example above and displaying a moderate exchange rate, I increases to a maximum in a similar fashion to the above but rapidly declines as τ_m approaches ∞.[10] Our NOESY data (Figure 3; all data points resulted from the integration of all off-diagonal -OH/H_2O resonances) clearly indicate that the title compound underwent some slow exchange-like process since I_{-OH/H_2O} stabilized as τ_m approached 1 s; K was also observed to vary with [H_2O]:[*N*-phenyl uronamide]. Rotating frame 2D Overhauser (ROESY)[7,8,16] enhancement H_1/H_5 cross peaks (Figure 4, upper spectrum) were negatively phased relative to the $C_{2,\ 3\ or\ 4\text{-}OH/H_2O}$ cross peaks (Figure 4, lower spectrum) thereby eliminating 1st order Overhauser effects as a possible explanation of our data. With regard to *N*-phenyl uronamide•4H_2O, we found (Table 1) that τ_c = ca. 0.54 ns via spin-lattice relaxation measurements at two fields. The calculated T_{1H}s were observed to diverge from the experimental an average of only 0.48%. Clearly, based upon the τ_c calculation and ROESY experiments the observed cross peaks between the solvating species, H_2O, and the title compound's hydroxyl ^1Hs were due to chemical exchange. Further support for a slow exchange mechanism is

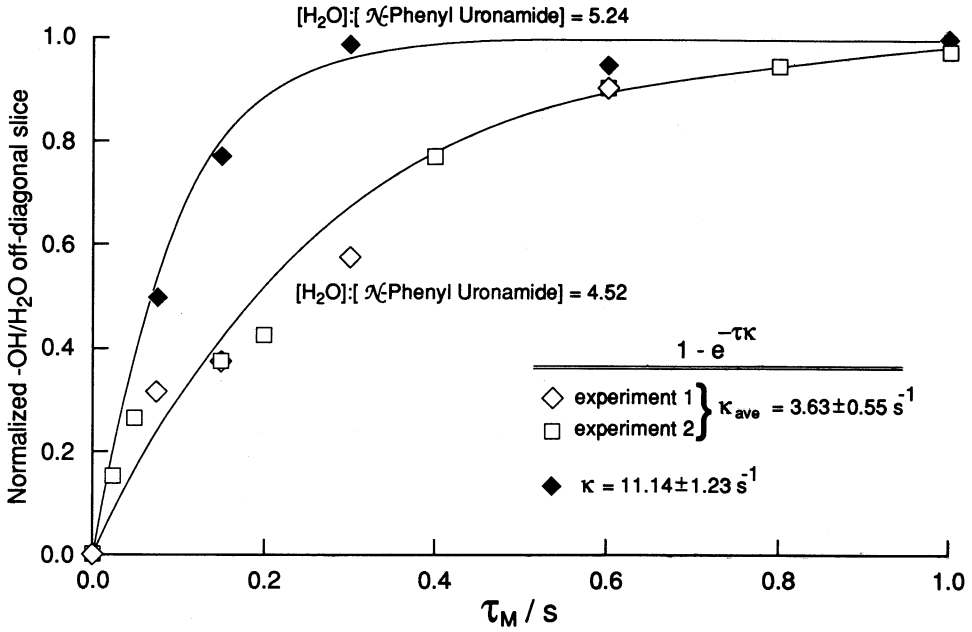

FIG. 3 Hydroxyl/H_2O cross peak area row slices plotted as a function of mixing time (τ_m) at 40 °C. Data were fit to a first order exponential rate equation.

presented in Figure 5 whereupon inversion recovery experiments were performed with specific irradiation at the H_2O's resonance frequency (T_{1sat}) and at an equivalent frequency off-set downfield (T_1) from the observed hydroxyl proton (C_4-OH, Figure 1). Based upon this measure of T_{1sat} and $\{I^+/I^\varnothing\}$ a κ_{sat} was found (see Experimental, Eqn 3) to be approximately 0.3 s^{-1}. The process of longitudinal relaxation without exchange effects would be most nearly represented by the T_{1sat} curve (open diamonds). The differences between these two treatments demonstrates the profound effect of exchange on the relaxation behavior of the title compound's -OH groups. Of course, other exchange-like processes can be eliminated as the foundation of our observations, *a priori*, since these occur mainly in macromolecular systems, glasses and other solid-like[12] milieu where τ_cs for molecular reorientation are on the order of tenfold longer.[11,13-15]

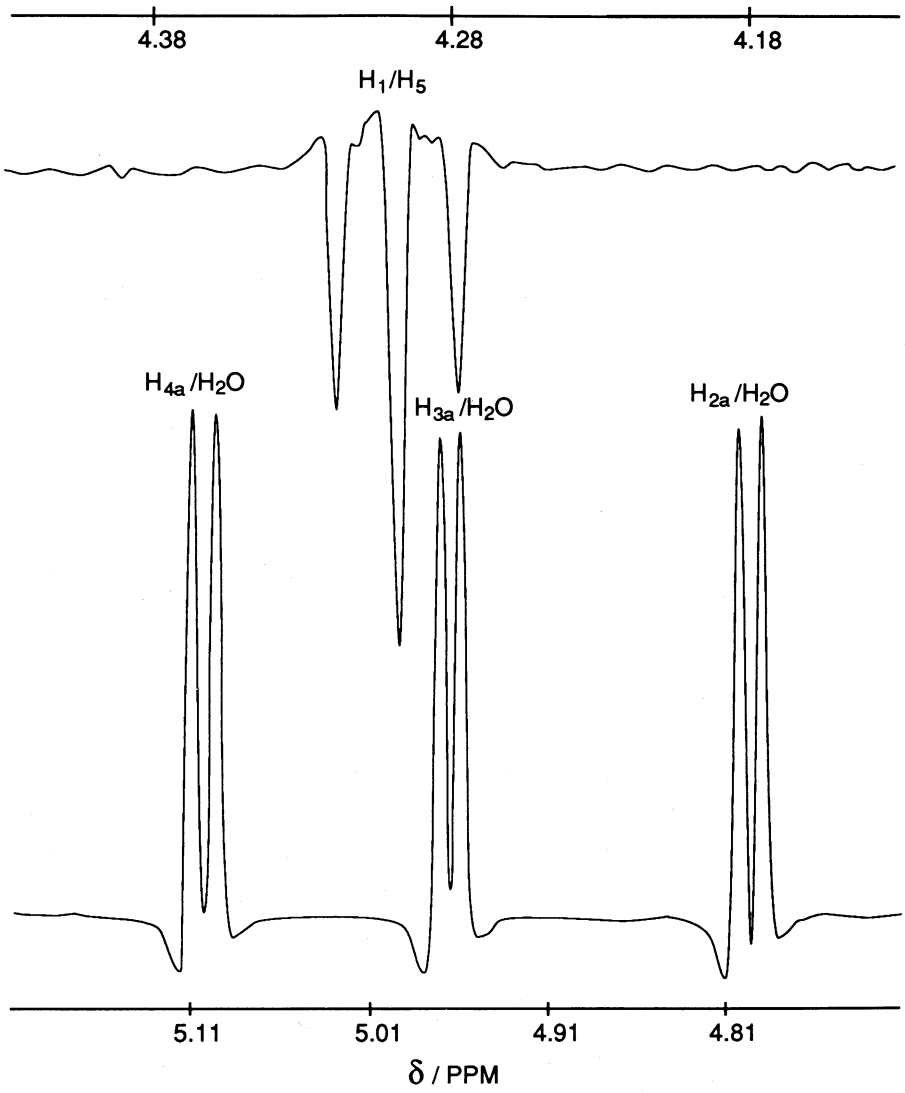

FIG. 4. H_1/H_5 and $-OH/H_2O$ ROESY (40 °C) cross peaks. The H_1/H_5 resonance (upper slice) displayed negative phasing which is indicative of the expected direct cross relaxation within the pyranose ring. The $-OH/H_2O$ resonances (lower slice) manifested positive phasing indicative of an exchange-like phenonenon.

Table 1. Proton NMR spectral assignments and spin-lattice relaxation times (observed, calculated and difference) at 2 fields. The correlation time (τ_c = 5.4 x 10^{-10} s) was based upon the static field dependence as shown in Eqn 4.

	T_{1H} / s[a] $\{\tau_c = 0.54$ ns$\}$					
	270 MHz			400 MHz		
$\delta\{^1H\}$	obs.[b]	calc.	Δ	obs.	calc.	Δ
4.5 {C_1-H}	0.25	0.32	0.07	0.61	0.57	-0.04
6.4 {C_1-NH}	0.33	0.32	-0.01	0.56	0.57	0.01
5.34 {C_2-OH}	0.84	0.83	-0.01	1.46	1.47	0.01
5.31 {C_3-OH}	0.84	0.81	-0.03	1.42	1.44	0.02
5.01 {C_4-OH}	0.85	0.79	-0.06	1.37	1.40	0.03
3.89 {C_5-H}	0.31	0.41	0.10	0.77	0.72	-0.05
7.66 {Ortho"}	1.09	1.05	-0.04	1.84	1.86	0.02
7.29 {Meta"}	1.11	1.19	0.08	2.15	2.10	-0.05
7.04 {Para"}	1.59	1.46	-0.13	2.50	2.57	0.07
6.76 {Ortho'}	0.66	0.67	0.01	1.20	1.19	-0.01
7.11 {Meta'}	1.02	1.04	0.02	1.86	1.86	0.00
6.64 {Para'}	1.05	1.14	0.09	2.08	2.08	0.00

a. 42 mM solution at 40 °C; see Eqn. 4 for τ_c-T_{1H} calculation

b. all T_{1H} calculation had ≤ 5 % calculation error.

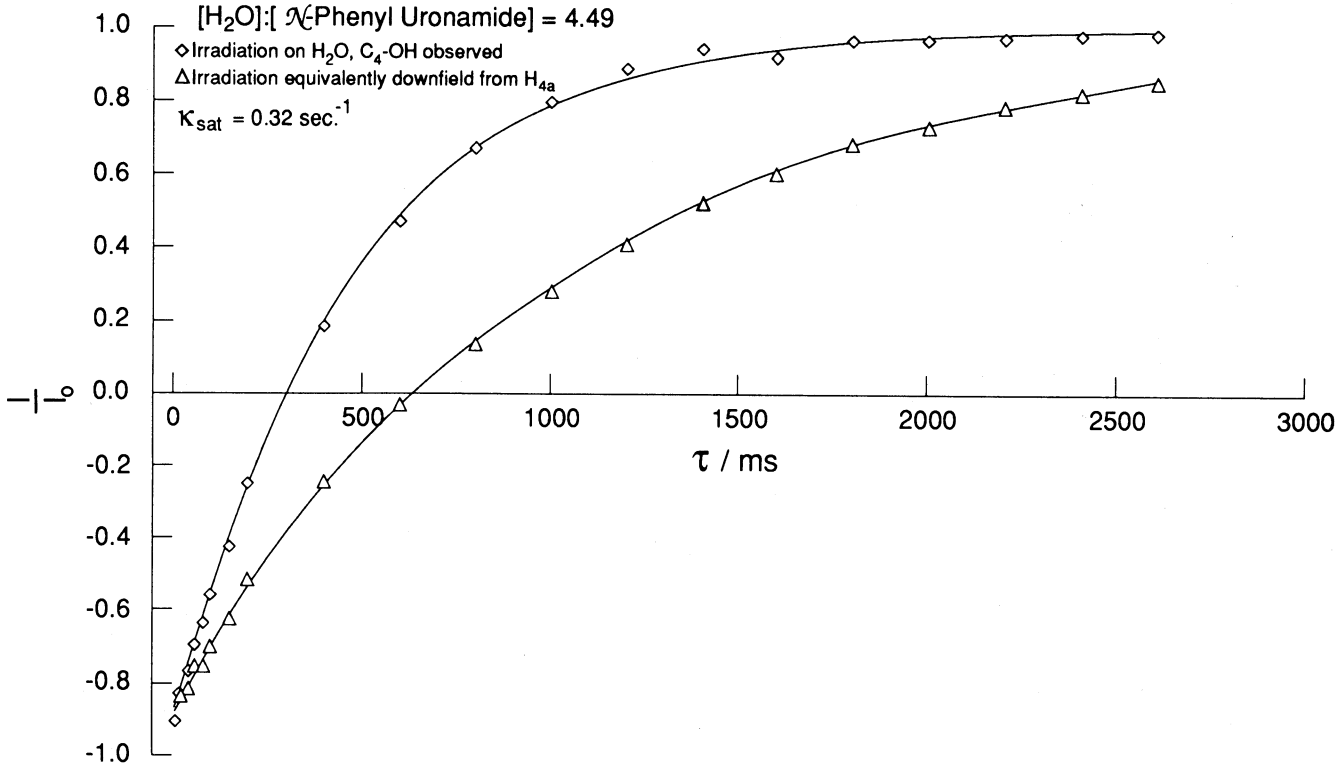

FIG. 5. Inversion recovery experiments on the title compound (C_4-OH observed) with irradiation 721.67 Hz upfield (e.g., on H_2O) from C_4-OH (diamonds) and with the same treatment 721.67 Hz downfield form C_4-OH (triangles). These experiments were performed at 40 °C and 400 MHz on a 400 mM solution of the title compound.

Interestingly the -OH/H_2O exponential exchange rate constant, κ, rose from 0.32 to 11.14 s^{-1} as the molar ratio of [H_2O]:[*N*-phenyl uronamide] increased only from ca. 4.5 to 5.2 (Figure 6). This latter finding indicated that the -OH/H_2O ^1H exchange rate constant, which has been shown to be affected by the translational diffusion of H_2O,[10] diminished as [H_2O] approached that which was inherently complexed in the crystalline structure ($C_{18}H_{20}O_5N_2 \cdot 4H_2O$) and was, presumably, tightly hydrogen bound to the -OH and -NH funtional groups when put into solution in DMSO. To further support this contention (Table 2), the anhydrous version of the title compound was compared to *N*-phenyl uronamide$\cdot 4H_2O$ with respect to chemical shift changes ($\Delta\delta$) upon dehydration. When no H_2O was present in the DMSO/*N*-phenyl uronamide solution a significant upfield shift on the resonance frequencies of all the exchangable (-OH $\Delta\delta$=86.33Hz; -NH $\Delta\delta$=67.84Hz) protons, relative to the methine protons (CH $\Delta\delta$=0.25Hz), was observed.

All these data are evidence that the H_2O in the system experienced the slowest exchange as the molar ratio of [H_2O]:[*N*-phenyl uronamide] approached 4. The translational diffusion of water, as measured by exchange, diminished dramatically as the molar ratio of H_2O:title compound approached that level bound in the crystalline structure and argues that the H_2O:*N*-phenyl uronamide complex has a relatively long lifetime in solution. At the above level of hydration most of the H_2O might not be available for translational diffusion thereby causing κ to diminish. However, as the [H_2O] increased beyond the capacity of *N*-phenyl uronamide to bind it, the average residence time would necessarily diminish due to the increased translational diffusion thus resulting in an elevation of κ as a function of [H_2O]. At [H_2O] levels much above $6H_2O$:1*N*-phenyl uronamide, the title compound breaks down as shown previously in H_2O:MeOH solutions.[2]

EXPERIMENTAL

Sample Preparation. The title compound was synthesized and purified as described previously[2] with minor modi-

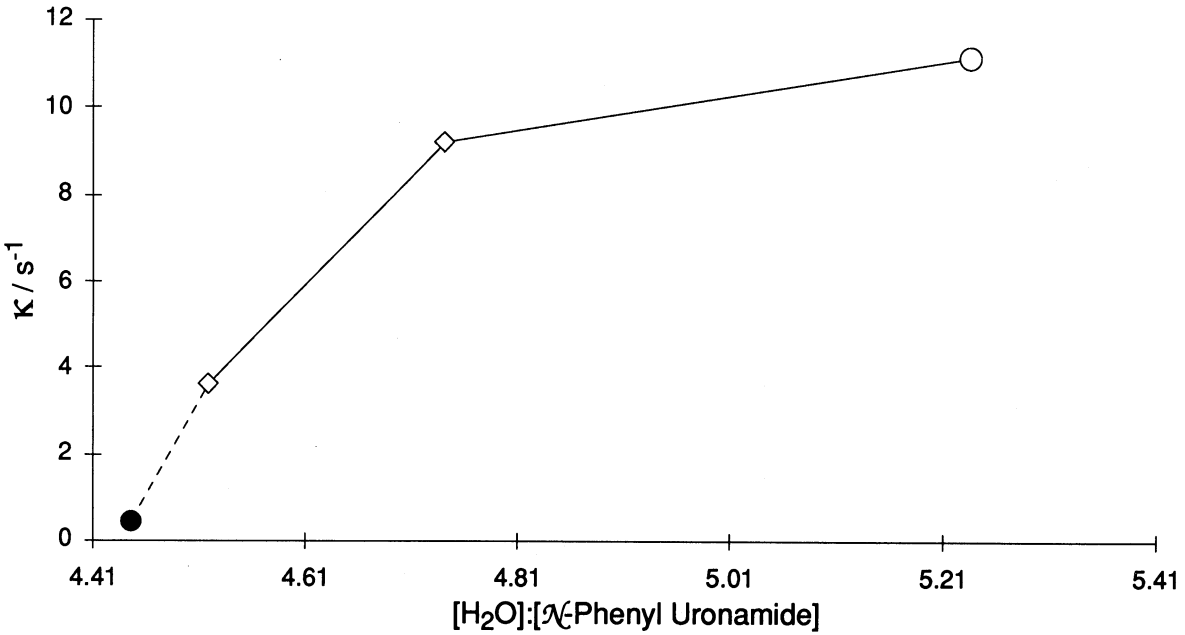

FIG. 6. Plot of ROESY (open circle), NOESY (diamonds) and T_{1sat}-calculated (closed circles) values of κ as a function of variable [H_2O]:[*N*-Phenyl Uronamide]. All experiments were performed at 400 MHz and 40 °C.

Table 2. Proton NMR spectral assignments for the title compound in the hydrated and dehydrated forms.

	δ/ppm		
	[water] : [N-Phenyl Uronamide]		
assignment	0:1	4:1	Δδ/Hz
Amide N-H[a]	9.91[b]	10.09[c]	72.00
Amine N-H	6.24	6.40	63.68
C_4-O-H	5.14	5.34	78.40
C_3-O-H	5.01	5.31	121.34
C_2-O-H	4.86	5.01	59.24
		$\bar{x}$ =	78.93
Ortho"	7.64	7.66	6.34
Meta"	7.29	7.29	0.00
Para"	7.05	7.04	-2.28
Ortho'	6.75	6.76	5.42
Meta'	7.11	7.11	0.00
Para'	6.64	6.64	0.00
C_1-H	4.50	4.50	0.00
C_2-H	3.31	3.29	-7.64
C_3-H	3.37	3.38	2.28
C_4-H	3.59	3.60	4.32
C_5-H	3.90	3.89	-5.74
		$\bar{x}$ =	0.25

a. 300 mM solution at 50 °C; made with "100%" DMSO-d_6 which was taken from a freshly-opened ampule

b. reacted with 2,2-dimethoxypropane prior to crystallization

c. ca. 4.41 H_2O molecules per molecule of *N*-phenyl (*N*-phenyl-β-**D**-glucopyranosyl-amine)uronamide

fications. For this purpose **D**-glucopyranuronic acid (1.5 g) was dissolved in 25 mL of H_2O whereupon aniline (2 mL) was added and the pH adjusted to 4.75 on a Radiometer[17] pH stat/titrator. Upon adding ca. 3 g of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) the pH stat was activated and 0.1N HCl added automatically to the reaction mixture to maintain the pH at ca. 4.75.[3-6] When no more titrant was needed to maintain a static pH the reaction was considered to be complete. At this point an insoluble, pasty, off-white precipitate had formed and was subsequently washed with H_2O to remove unreacted aniline or EDC. Excess H_2O was removed by washing the precipitate with chilled EtOH. The **D**-glucopyranuronic acid derivative was then dissolved in hot EtOH; 2-4 mm needle-like crystals formed overnight at room temperature. For crystallization of the anhydrous form, the above procedure was repeated exactly except that a small amount of 2,2-dimethoxypropane (≤ 10 mole %) was added to the EtOH to react with unwanted H_2O (e.g., H_2O + 2,2-dimethoxypropane $\xrightarrow[\Delta]{}$ 2MeOH + acetone).

NMR samples were prepared in a dry box with DMSO-d_6 (≥99.5 atom % ^2H) which had been previously stored several days with molecular sieve pellets under dry N_2.[18] Several DMSO-d_6-washed molecular sieve pellets were kept in the 5 mm NMR tubes to maintain the sample in a relatively dry state; the NMR tubes were closed and wrapped with parafilm to assist in the exclusion of extraneous H_2O vapor. The samples were stored at ca. 3 °C and underwent no obvious degradative process, such as pyranose ring opening and associated Amadori rearrangement or loss of the C_1 amine functionality.

NMR Spectroscopy. Preceding all NMR experiments, the 90° pulse was determined for each experimental condition, such as variable temperature or concentration of title compound, utilizing standard methods.[19] All NOESY spectra were collected on a JEOL GX-400 NMR spectrometer system operated at ca. 400 MHz (B_o = 9.40 T) for ^1H using 5mm probes. Computer line broadening was selected to be approximately equal to the digital resolution. These experiments were ac-

quired using a matrix of 128 x 1024 (t_1 x t_2), 256 x 2048 after zero-filling, complex data points which represented a spectral width of 953.1 Hz for either dimension. For each t_1 spectrum collected, 16 transients were acquired. A sine-bell apodization function was used to process these data. All quantitative 2D Overhauser enhancement matrices were processed without symmetrization. Specific details, such as concentration of the title compound and temperature, are provided in each figure or table caption. All ROESY[2] data were collected using a JEOL GSX-400 NMR spectrometer. The proton full-power 90° pulse was 10.5 μs. Acquisition data sets consisted of 2048 complex points for t_2 and 64 acquisitions for each t_1 data set. A spin-lock field of 3 kHz, 1 kHz off-resonance from the average chemical shifts of the residual H_2O protons and the -OHs, was used for τ_ms of 0.75, 0.2, 0.4 and 0.6 s. The data sets were zero-filled to 4096 t_2 points and 2048 points for t_1. A phase-shifted sine-bell was used as the window function. All the -OH resonances ($H_{2a\rightarrow 4a}$) were integrated for fitting to an exponential function (eqn 1) using a modified Gauss-Newton microcomputer program developed in this laboratory.

Proton T_1 inversion recovery experiments, shown in Table 1, were performed on JEOL NMR spectrometers operated at either 400 or 270 MHz (B_o = 9.40 or 6.34 T). Each τ value was signal averaged for 64 acquisitions with 16 dummy scans. The ^1H NMR experiments shown in Table 2 were performed on a JEOL GX-400 NMR spectrometer system using 5mm ^1H fixed probes. T_{1sat} experiments[20-22] were performed identically to the above 400 MHz T_1 experiments except that the H_2O resonance was irradiated 721.67 Hz upfield from the C_4-OH (H_{4a}) resonance. All peak intensity data were fit to an exponential function, $I_i = I_o\{1-2\exp[-(\tau_i - \tau_o)/T_1]\}$; in this expression $I_i = -I_o$ ($I_i = I_o$ as $\tau\rightarrow\infty$) at τ_o. The T_{1sat}-associated pseudo first-order rate constant (κ_{sat}) calculation was performed as demonstrated in Eqn 3,

$$\kappa_{sat} = \frac{1}{T_{1sat}} - \frac{\{I^+/I^\varnothing\}}{T_{1sat}} \qquad (3);$$

$\{I^{+}/I^{\emptyset}\}$ is the ratio of hydroxyl resonance integrals with irradiation on the H_2O resonance and 721.67 Hz downfield, respectively. T_{1sat} is the normal T_1 measurement but with spin saturation of H_2O. The overall molecular correlation time (τ_c = 5.4 x 10^{-10} s; Table 1) and calculated T_{1i}'s were estimated as shown in Eqn 4

$$\frac{1}{T_{1i}} = \frac{3}{10}\gamma^4\left[\frac{h}{2\pi}\right]^2 \sum_j \frac{1}{r_{ij}^6}\left\{\frac{\tau_c}{1 + (\nu_o\tau_c)^2} + \frac{4\tau_c}{1 + (2\nu_o\tau_c)^2}\right\} \qquad (4);$$

in this relationship ν_o was 270 or 400 MHz. The interproton distance parameter, r_{ij}, was assumed to be ca. 2 Å; it was further assumed that the relaxation process involved 2 protons. Due to the reciprocal 6^{th} power influence of r_{ij} on T_{1i} the interproton distance parameter has only a minor effect on the calculated T_{1i}s.

ACKNOWLEDGEMENTS

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17. Reference to brand or firm name does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

18. The DMSO contained, except when specifically specified (e.g., when utilizing "100%" DMSO-d_6 which was taken from a freshly-opened ampule), ca. 30 mM H_2O even in the presence of "dry" molecular sieves.

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